Molecular Medicine

Piwil 2 Expression Is Correlated with Disease-Specific and Progression-Free Survival of Chemotherapy-Treated Bladder Cancer Patients

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Piwi-like 2 (Piwil 2) belongs to the family of Argonaute genes/proteins. The expression of Piwil 2 is associated with stem cells. A role in tumorigenesis and/or tumor progression is proposed for different cancers but not yet for bladder cancer (BCa). We investigated Piwil 2 expression by immunohistochemistry in a cohort of 202 BCa patients treated by cystectomy and adjuvant chemotherapy. The association between Piwil 2 expression and disease-specific (DSS) or progression-free survival (PFS) was calculated using Kaplan-Meier analyses and univariate/multivariate Cox regression hazard models. In a multivariate Cox regression analysis, Piwil 2 expression, either in the cytoplasm or the nucleus, was significantly associated with DSS and PFS. A weak cytoplasmic staining pattern was associated with poor DSS and tumor progression (relative risk (RR) = 2.7, $P = 0.004$, and RR = 2.4, $P = 0.027$). Likewise, absent nuclear Piwil 2 immunoreactivity was associated with poor DSS and tumor progression (RR = 2.3, $P = 0.023$, and RR = 2.2, $P = 0.022$). BCa patients whose tumors exhibited a combination of weak cytoplasmic and absent nuclear immunoreactivity had a 6-fold increased risk of tumor-related death ($P = 0.005$) compared with patients with strong expression. Considering only patients with high-grade G3 tumors, a 7.8-fold risk of tumor-associated death and a 3.6-fold risk of tumor progression were detected independently of the histologic tumor subtype or the chemotherapy regimen. In summary, a combination of weak cytoplasmic and absent nuclear expression of Piwil 2 is significantly associated with an increased risk of DSS and tumor progression. This indicates that Piwil 2 could be a valuable prognostic marker for high-risk BCa patients.

INTRODUCTION

Bladder cancer (BCa) is the ninth most commonly diagnosed cancer and the 13th leading cause of cancer-related death worldwide (1). Clinical management of BCa (2) and the etiology (3–5) and diagnostic, prognostic or predictive biomarkers for BCa have been described extensively (2) (reviewed in [6,7]). While there are treatment options available for both superficial and invasive BCa, metastatic disease still presents a serious clinical problem with limited therapeutic options. Therefore, there is an urgent need to identify additional useful biomarkers in BCa.

Piwi-like 2 (*PIWIL2*) is a member of the *Piwi-like* (P-element-induced wimpy testis) gene family, a subclass of the Argonaute gene family (8) (reviewed in [9]). These genes are characterized by their homology and the occurrence of Piwi Argonaute Zwille (PAZ) and Piwi domains (10) (reviewed in [11]). *Piwi-like* genes are essential for stem cell maintenance and self-renewal in multicellular organisms ranging from plants to humans (12,13). Piwi proteins play important roles in stem cell self-renewal, spermatogenesis, transposon and RNA silencing, translational regulation and chromatin remodeling in various organisms (9,14). Piwi proteins can bind specifically to 24- to 32-nucleotide–long noncoding RNAs (Piwi-interacting RNA

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Table 1. Clinicopathological data and cytoplasmic Piwil 2 protein expression.[a]

		Piwil 2 cytoplasmic staining			
	Total	Negative	Weak	Moderate	Strong
IRS	0	1–2	3–4	6–12	
N	202	37	53	55	57
Age					
Range	35–77	48–75	38–77	35–75	41–74
Mean	61.7	62.3	62.6	61.7	60.4
Median	61.9	62.9	62.9	62.6	61.0
Sex					
Female	46	5	15	8	18
Male	156	32	38	47	39
pT					
pT1	5	2	2	0	1
pT2	25	4	10	4	7
pT3	135	27	31	37	40
pT4	37	4	10	14	9
pN					
N0	89	13	22	27	27
N1	41	6	10	13	12
N2	70	17	21	14	18
N3	1	0	0	1	0
Unknown	1	1	0	0	0
Grade					
Grade 2	26	7	7	3	9
Grade 3	176	30	46	52	48
Histotype					
UC	179	31	45	47	56
PUC	14	4	6	3	1
MPC	9	2	2	5	0
Chemotherapy					
Gem/Cis	45	10	13	15	7
Mono Gem	18	4	4	6	4
M-VEC	66	9	17	15	25
CM	73	14	19	19	21
DSS					
Alive	136	29	31	38	38
Dead	66	8	22	17	19

[a]UC, conventional urothelial carcinoma; PUC, plasmacytoid urothelial carcinoma; MPC, micropapillary urothelial carcinoma; Gem, gemcitabine; Cis, cisplatin; M-VEC, methotrexate, vinblastine, epirubicin, cisplatin; CM, cisplatin, methotrexate.

[piRNA]), and in this way, they occupy the interface between stem cell and small RNA biology (15).

The *PIWIL2* gene is located on chromosome 8 (8p21.3) and comprises 23 exons. It encodes a protein of 973 amino acids with a molecular weight of 110 kDa. Expression of the *PIWIL2* gene was verified in different human tumors, such as testicular germ cell tumors; prostate, breast, gastrointestinal, ovarian and endometrial cancer; leukemia; and murine breast tumors, rhabdomyosarcomas and medulloblastomas (16–18) (reviewed in [9,15]). Overexpression of the Piwi 2 protein is also correlated with the occurrence of colon cancer (19). Piwi 2 can be detected by immunohistochemistry (IHC) in various stages of squamous cell carcinomas and adenocarcinomas of human cervical and breast cancer (20–23). Piwi 2 was also detected in some metaplastic epithelial cells as well as histologically normal tissues adjacent to malignant lesions in cervical and mammary carcinomas (20,21). Recently, a stronger expression of Piwi 2 in the primary tumor and metastatic tissues of ovarian cancer compared with adjacent normal tissue was reported (24), and it varied depending on the differentiation subtype of testicular germ cell tumors (TGCT) (18). Several studies displayed in the Oncomine gene browser (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) show a rather low expression of *PIWIL2* mRNA in BCa tissues. Furthermore, mRNA expression was very low or even undetectable in all examined BCa cell lines and the expression of *PIWIL2* in benign or malignant bladder tissue was estimated to be less than 0.5% of the level in control testicular tissue (25). So far, no study has reported on Piwi 2 protein expression in BCa, and therefore its association with DSS or PFS has not been investigated yet.

MATERIALS AND METHODS

Patients and Tumor Materials

Tissue microarrays (TMA) with formalin-fixed and paraffin-embedded tumor samples of 202 BCa patients were used in this study. The majority of the patients were treated within the randomized AUO-AB05/95 clinical trial by radical cystectomy and adjuvant cisplatin-based chemotherapy. Tumor histology was reviewed retrospectively by an experienced uropathologist (A Hartmann). The study population comprising a cohort of BCa patients with either ≥pT3 or ≥pN1 (any pT) tumors and the details of the AUO-AB05/95 trial have been reported previously (26). The research carried out on human subjects is in compliance with the Helsinki Declaration. An overview of the clinicopathologic parameters of the patients included in this study is shown in Tables 1 and 2.

Immunohistochemistry

We would like to thank JX Gao (Ohio State University Medical Center, Colum-

bus, Ohio, USA) for kindly providing the anti-Piwil 2 antibody described in detail in (22) For the study of Piwil 2 protein expression, a manual IHC protocol was applied as previously described (27). Briefly, after heat pretreatment at 120°C for 5 min with Tris-EDTA (TE)-buffer pH 9 and peroxidase blocking (Dako, Glostrup, Denmark), primary antibodies against Piwil 2 (polyclonal rabbit IgG, 1:500) (23) were applied for 30 min. After incubation with a respective horseradish peroxidase (HRP)-labeled secondary antibody polymer (EnVision, Dako) for 30 min, a diaminobenzidine (DAB) substrate chromogen solution (Dako) was added for 10 min. The slides were counterstained for 1 min with hematoxylin (Merck, Darmstadt, Germany). Between all of the steps, the slides were washed with buffer from Dako and all of the incubation steps were performed at room temperature. Stained specimens were viewed at an objective magnification of 100× and 200× by an experienced uropathologist (A Hartmann). Expression of Piwil 2 was evaluated separately in the nucleus and in the cytoplasm by assessing the percentage of stained tumor cells and the staining intensity semiquantitatively. To calculate an immunoreactive score (IRS 0–12) (28), the scores for the percentages of positive cells and the scores for expression intensities were multiplied. The percentage of positive cells was rated as follows: 1, 1%–10% positive cells; 2, 11%–50%; 3, 51%–80%; and 4, >80% positive cells. Staining intensity was scored as 0, negative; 1, weak; 2, moderate, and 3, strong. We separated the BCa patients into groups according to their cytoplasmic or nuclear expression of Piwil 2 in 25% percentile groups: group 1, IRS 0; group 2, IRS 1 to 2; group 3, IRS 3 to 4; group 4, IRS 6 to 12. As a negative control, slides without addition of primary antibody were included for each staining.

Statistical Analyses

The associations between IHC staining patterns were analyzed using the Spearman rank test, and the associations between IHC and clinical data were calculated using the χ^2 test. The associations of the expression of Piwil 2 with disease-specific survival (DSS) or progression-free survival (PFS) were determined in univariate (Kaplan-Meier analysis and Cox regression hazard models) and multivariate analyses (Cox regression hazard models, adjusted to histological subtype, tumor therapy, tumor grade, tumor stage, sex and age). A p value of less than 0.05 was considered statistically significant. Statistical analyses were performed with the SPSS 19.0 software package (SPSS [IBM, Armonk, NY, USA]).

RESULTS

Piwil 2 Expression in Bladder Cancer Patients

The expression of Piwil 2 was determined in the cytoplasm and nucleus of 202 BCa specimens by immunohistochemistry. The expression was negative in 37 cases, weak in 53 cases, moderate in 55 cases and strong in 57 cases in the cytoplasm, and it was negative in 57, weak in 50, moderate in 46 and strong in 49 cases in the nucleus (Tables 1 and 2; Figure 1). There was no correlation between staining in the cytoplasm or the

Table 2. Clinicopathological data and nuclear Piwil 2 protein expression.

		Piwil 2 nuclear staining			
	Total	Negative	Weak	Moderate	Strong
IRS	0	1–2	3–4	6–12	
N	202	57	50	46	49
Age					
Range	35–77	44–62	35–77	43–75	38–71
Mean	61.7	62.0	62.1	62.8	59.8
Median	61.9	61.9	61.6	64.1	61.9
Sex					
Female	46	10	14	12	10
Male	156	47	36	34	39
pT					
pT1	5	1	2	2	0
pT2	25	10	4	7	4
pT3	135	39	32	28	36
pT4	37	7	12	9	9
pN					
N0	89	24	22	19	24
N1	41	11	7	10	13
N2	70	22	20	16	12
N3	1	0	1	0	0
Unknown	1	0	0	1	0
Grade					
Grade 2	26	10	4	5	7
Grade 3	176	47	46	41	42
Histotype					
UC	179	50	46	39	44
PUC	14	6	4	2	2
MPC	9	1	0	5	3
Chemotherapy					
Gem/Cis	45	14	10	11	10
Mono Gem	18	8	4	4	2
M-VEC	66	20	14	16	16
CM	73	15	22	15	21
DSS					
Alive	136	36	34	30	36
Dead	66	21	16	16	13

cytopl. IRS=0, nucl. IRS=0

cytopl. IRS=0, nucl. IRS=8

cytopl. IRS=0, nucl. IRS=12

cytopl. IRS=1, nucl. IRS=0

cytopl. IRS=2, nucl. IRS=12

cytopl. IRS=4, nucl. IRS=4

cytopl. IRS=4, nucl. IRS=12

cytopl. IRS=12, nucl. IRS=0

Figure 1. Examples of immunohistochemical staining for Piwil 2 protein in BCa samples. All photos are magnified 400×; cytopl., cytoplasmic; nucl., nuclear.

nucleus (Spearman rank test, data not shown).

Association of Piwil 2 Protein Expression with Clinicopathological Data

Piwil 2 protein expression and clinicopathological parameters, such as age, sex, tumor stage, tumor grade, histologic subtype or type of chemotherapy, showed no association with each other (χ^2 test, data not shown).

Association of Piwil 2 Protein Expression with DSS of BCa Patients

At first, we studied whether Piwil 2 protein expression was correlated with the survival of BCa patients. We separated the patients according to the 25% percentiles of Piwil 2 protein expression into four groups: negative (IRS = 0), weak (IRS = 1–2), moderate (IRS = 3–4) and strong (IRS = 6–12) Piwil 2 staining.

Kaplan-Meier analysis of DSS revealed the worst prognosis in patients with

weak cytoplasmic Piwil 2 protein expression in their tumors (42.8 months, 95% CI: 30.3–55.4 months, P = 0.022; Table 3) compared with the other BCa patients and for BCa patients with negative nuclear staining (39.4 months, 95% CI: 30.7–48.1 months; not significant) compared with the other BCa patients.

To estimate the risk for disease-specific death in relation to Piwil 2 protein expression, univariate and multivariate Cox regression analyses (adjusted for clinical parameters, including histologic tumor subtype, tumor therapy, tumor grade, tumor stage, sex and age) were performed. A weak cytoplasmic Piwil 2 staining was significantly associated with a 2.2-fold risk (univariate, 95% CI: 1.2–4.0; P = 0.014) and a 2.7-fold risk (multivariate, 95% CI: 1.4–5.3; P = 0.004) for tumor-related death compared with patients with a strong cytoplasmic Piwil 2 expression in their tumors. A negative nuclear staining for Piwil 2 was significantly correlated with a 2.4-fold (univariate, 95% CI: 1.2–4.8; P = 0.017) and a 2.3-fold risk (multivariate, 95% CI: 1.1–4.8; P = 0.023; Table 3) compared with patients with a strong nuclear Piwil 2 expression in their tumors (Table 3).

Because we could demonstrate that the staining patterns in the cytoplasm and nucleus were not correlated with each other, we decided to regard them as independent risk factors and combined the cytoplasmic and nuclear Piwil 2 staining scores and analyzed their correlation with DSS. Patients expressing weak cytoplasmic and negative nuclear staining patterns of Piwil 2 had a mean survival time of 23.3 months (95% CI: 16.6–30.0 months; Table 3), whereas patients with strong cytoplasmic and nuclear expression showed a mean survival time of 78.1 months (95% CI: 63.4–92.7 months), which was significantly different (P = 0.007; log-rank test; Table 3; Figure 2A). In univariate and multivariate Cox regression models, patients with weak cytoplasmic and negative nuclear staining patterns of Piwil 2 had a significantly increased risk of tumor-related death compared with patients with strong expression of Piwil 2

Table 3. Association between cytoplasmic, nuclear and the combination of cytoplasmic/nuclear expression of Piwil 2 and DSS in BCa patients.

	Group	N	Univariate Kaplan-Meier, months (95% CI)	*P*	Univariate Cox regression, RR (95% CI)	*P*	Multivariate Cox regression,[a] RR (95% CI)	*P*
All tumors		202						
Cytoplasmic	IRS 0	37	73.4 (57.9–89.3)		0.8 (0.3–1.8)	0.609	0.9 (0.4–2.2)	
	IRS 1–2	53	42.8 (30.3–55.4)	0.022	2.2 (1.2–4.0)	0.014	2.7 (1.4–5.3)	0.004
	IRS 3–4	55	62.7 (50.6–74.5)		1.1 (0.6–2.2)	0.688	1.1 (0.5–2.2)	0.576
	IRS 6–12	57	63.8 (53.2–74.5)		Reference		Reference	
Nuclear	IRS 0	57	39.4 (30.7–48.1)	0.103	2.4 (1.2–4.8)	0.017	2.3 (1.1–4.8)	0.023
	IRS 1–2	50	63.7 (50.0–77.4)		1.5 (0.7–3.1)	0.308	1.4 (0.7–3.2)	0.305
	IRS 3–4	46	57.8 (44.6–71.0)		1.6 (0.8–3.3)	0.295	1.5 (0.7–3.3)	0.277
	IRS 6–12	49	69.2 (57.9–80.5)		Reference		Reference	
Combination cytoplasmic/nuclear	IRS 1–2/IRS 0	17	23.3 (16.6–30.0)	0.007	5.9 (1.7–20.0)	0.004	6.0 (1.7–21.0)	0.005
	Others	168	61.9 (54.3–69.7)		2.4 (0.8–6.6)	0.098	2.4 (0.8–6.9)	0.104
	Both IRS 6–12	17	78.1 (63.4–92.7)		Reference		Reference	
Only grade 3 tumors		176						
Cytoplasmic	IRS 0	30	69.9 (52.6–87.1)		1.1 (05–2.6)	0.846	1.2 (0.5–2.9)	0.736
	IRS 1–2	46	35.3 (22.7–47.8)	0.001	3.1 (1.6–6.2)	0.001	3.5 (1.7–7.1)	0.001
	IRS 3–4	52	66.5 (54.3–78.7)		1.2 (0.6–2.4)	0.708	1.1 (0.5–2.4)	0.769
	IRS 6–12	48	66.8 (55.7–77.9)		Reference		Reference	
Nuclear	IRS 0	47	38.6 (29.5–47.6)	0.096	2.6 (1.2–5.7)	0.016	2.5 (1.1–5.5)	0.026
	IRS 1–2	46	63.6 (49.4–77.9)		1.6 (0.7–3.6)	0.239	1.7 (0.7–3.9)	0.203
	IRS 3–4	41	59.6 (44.9–74.3)		1.5 (0.7–3.8)	0.226	2.0 (0.8–4.7)	0.113
	IRS 6–12	42	71.2 (59.2–83.2)		Reference		Reference	
Combination cytoplasmic/nuclear	IRS 1–2/IRS 0	17	23.3 (16.6–30.0)	0.004	7.6 (1.9–29.5)	0.016	7.8 (1.9–31.1)	0.004
	Others	143	62.1 (53.7–70.5)		3.1 (0.9–10.2)	0.057	3.1 (0.9–10.1)	0.067
	Both IRS 6–12	16	82.7 (70.1–95.3)		Reference		Reference	

[a]Multivariate Cox regression analysis adjusted to tumor histology, tumor therapy, tumor grade, tumor stage, sex, and age; in grade 3 tumors adjusted to tumor histology, tumor therapy, tumor stage, sex and age. The differences between univariate analyses are determined by censored cases before the events occurred in the groups (what is considered directly in the Kaplan-Meier analysis), and in the Kaplan-Meier analysis, the calculation covers all of the groups in one step, whereas in the univariate Cox regression analysis, it compares single groups with a reference group.

protein (relative risk [RR] = 5.9, 95% CI: 1.7–20.0; *P* = 0.004; and RR = 6.0, 95% CI: 1.7–21.0; *P* = 0.005; Table 3).

Association of Piwil 2 Protein Expression with DSS of High-Grade (G3) Bladder Cancer Patients

Analogous to the analyses presented previously, we performed a subgroup analysis of high-grade G3 BCa patients. Here, the association between protein expression of Piwil 2 either in the cytoplasm or the nucleus and DSS was even more distinct (Table 3).

Kaplan-Meier analysis of DSS identified the poorest prognosis in patients with weak cytoplasmic Piwil 2 staining patterns in their tumors (35.3 months, 95% CI: 22.7–47.8 months; *P* = 0.001)

(Table 3) compared with the other BCa patients and in patients with a negative nuclear staining at 38.6 months (95% CI: 29.5–47.6 months, not significant) compared with the other BCa patients.

The risk for tumor-specific death of Piwil 2 protein expression was again determined in univariate and multivariate Cox regression analyses (adjusted for tumor histology, tumor therapy, tumor stage, sex and age). Weak cytoplasmic Piwil 2 expression was significantly associated with a 3.1-fold risk (univariate, 95% CI: 1.6–6.2; *P* = 0.001) and a 3.5-fold risk (multivariate, 95% CI: 1.7–7.1; *P* = 0.001) for tumor-related death compared with patients with a strong expression in their tumors. A negative nuclear staining pattern for Piwil 2 was significantly cor-

related with a 2.6-fold (univariate, 95% CI: 1.2–5.7; *P* = 0.016) and a 2.5-fold risk (multivariate, 95% CI: 1.1–5.5; *P* = 0.026; Table 3) compared with patients with strong expression in their tumors.

Next, we studied the combined cytoplasmic and nuclear Piwil 2 protein expression and their correlation with DSS. Grade 3 BCa patients showing weak cytoplasmic and negative nuclear staining of Piwil 2 had a mean survival of 23.3 months (95% CI: 16.6–30.0 months; Table 3), whereas patients with strong cytoplasmic and nuclear staining exhibited a mean survival of 82.7 months (95% CI: 70.1–95.3 months), which was significantly different (*P* = 0.004; log-rank test; Table 3; Figure 2A). In univariate and multivariate Cox regression hazard mod-

A Disease-Specific Survival

All BCa Patients
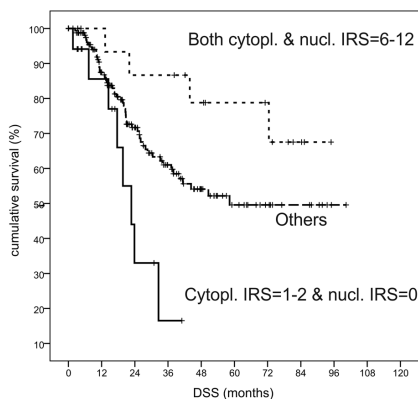
Only High-Risk Grade 3 BCa Patients
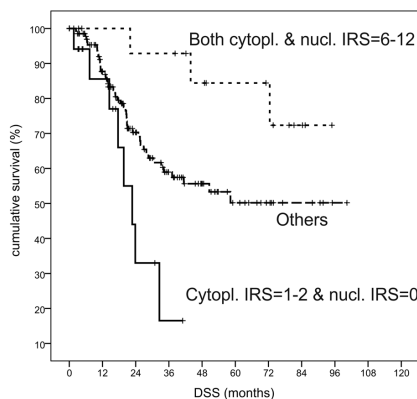
B Progression-Free Survival

All BCa Patients
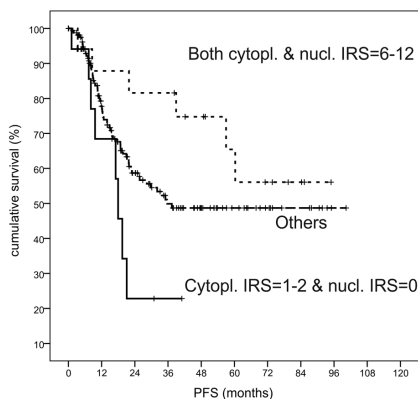
Only High-Risk Grade 3 BCa Patients
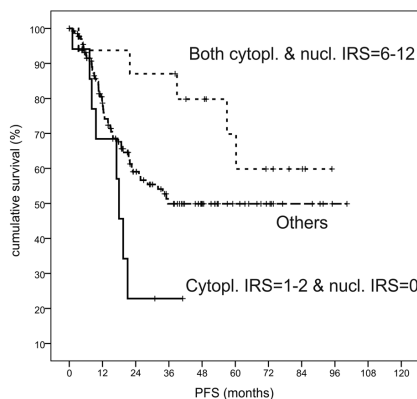
Figure 2. Kaplan-Meier analysis: association of combined Piwil 2 protein expression in the cytoplasm and the nucleus with DSS and PFS.

els, patients with weak cytoplasmic and negative nuclear staining of Piwil 2 had a significantly increased risk of tumor-related death compared with patients with high expression of Piwil 2 protein (RR = 7.6, 95% CI: 1.9–29.5; $P = 0.016$ and RR = 7.8, 95% CI: 1.9–31.1; $P = 0.004$; Table 3).

Association of Piwil 2 Protein Expression with PFS

In a Kaplan-Meier analysis, a shorter PFS was somewhat associated with a weak expression of Piwil 2 in the cytoplasm (42.4 months, 95% CI: 28.9–55.8 months) or a negative nuclear staining of Piwil 2 (34 months, 95 CI: 25.4–42.6 months) compared with other BCa pa-

tients, but these associations were not significant. However, in the univariate and multivariate Cox regression analyses, a significant association for both staining patterns with an approximate 2-fold increased risk of tumor progression was observed. A weak cytoplasmic Piwil 2 staining was associated with a 2.2-fold (univariate, 95% CI: 1.0–4.6, $P = 0.040$) or a 2.4-fold (multivariate, 95% CI: 1.1–5.2; $P = 0.027$) increased risk of tumor progression (Table 4). A negative nuclear Piwil 2 staining was comparably associated with a 2.1-fold (univariate, 95% CI: 1.1–3.9; $P = 0.024$) and a 2.2-fold (multivariate, 95% CI: 1.1–4.3; $P = 0.022$) increased risk of tumor progression (Table 4). When com-

bining cytoplasmic and nuclear staining patterns, patients with a weak cytoplasmic and negative nuclear Piwil 2 staining showed disease progression after an average of 20.2 months (12.6–27.8 months), whereas patients with a strong cytoplasmic and nuclear staining experienced tumor progression on average after 68.9 months (52.2–85.7 months), but this difference was not significant ($P = 0.067$; Figure 2B). However, in a univariate Cox regression analysis, a significant 3.4-fold increased risk of tumor progression was observed in the group with a weak cytoplasmic and negative nuclear staining compared with the group with strong staining in both compartments ($P = 0.025$).

Association of Piwil 2 Protein Expression with PFS of High-Grade (G3) Bladder Cancer Patients

In a Kaplan-Meier analysis, when considering only high-grade G3 patients, a significant association between weak cytoplasmic staining and a shorter PFS (35.3 months, 95% CI: 21.1–49.4 months; $P = 0.041$) was observed compared with other BCa patients. This association could also be detected in the univariate and multivariate Cox regression analyses, that is, a weak cytoplasmic Piwil 2 staining was, in both comparisons, associated with a 2.5-fold increased risk of tumor progression ($P = 0.024$ and $P = 0.027$, Table 4). In a Kaplan-Meier analysis, the shortest mean survival was detected in the group with negative nuclear staining (33.9 months, 95% CI: 24.8–43.1), and it was not significantly different from those of the other BCa patients. However, in univariate and multivariate Cox regression analyses, a significant 2.1-fold (univariate, 95% CI: 1.0–4.2; $P = 0.035$) and a 2.2-fold (multivariate, 95% CI: 1.1–4.6; $P = 0.033$) increased risk of tumor progression was observed for the patients with a negative nuclear staining. The high-grade G3 BCa patients could therefore be separated into different progression risk groups according to their Piwil 2 expression. The group with weak cytoplasmic and negative nuclear stain-

Table 4. Association between cytoplasmic, nuclear and the combination of cytoplasmic/nuclear expression of Piwil 2 and PFS in BCa patients.

	Group	N[b]	Univariate Kaplan-Meier, months (95% CI)	P	Univariate Cox regression, RR (95% CI)	P	Multivariate Cox regression,[a] RR (95% CI)	P
All tumors		201						
Cytoplasmic	IRS 0	37	70.1 (54.5–85.7)		Reference		Reference	
	IRS 1–2	53	42.4 (28.9–55.8)	0.190	2.2 (1.0–4.6)	0.040	2.4 (1.1–5.2)	0.027
	IRS 3–4	54	58.4 (45.9–70.9)		1.4 (0.7–3.1)	0.366	1.3 (0.6–2.9)	0.47
	IRS 6–12	57	52.9 (42.1–63.7)		1.5 (0.7–3.2)	0.241	1.6 (0.7–3.4)	0.26
Nuclear	IRS 0	57	34.0 (25.4–42.6)	0.125	2.1 (1.1–3.9)	0.024	2.2 (1.1–4.3)	0.022
	IRS 1–2	50	60.9 (47.1–74.8)		1.3 (0.6–2.6)	0.441	1.4 (0.7–2.8)	0.366
	IRS 3–4	45	54.1 (40.7–67.4)		1.4 (0.7–2.8)	0.325	1.4 (0.7–2.8)	0.388
	IRS 6–12	49	62.8 (50.7–75.0)		Reference		Reference	
Combination cytoplasmic/nuclear	IRS 1–2/IRS 0	17	20.2 (12.6–27.8)	0.067	3.4 (1.2–9.9)	0.025	3.0 (0.9–9.3)	0.051
	Others	167	57.1 (49.4–64.7)		1.7 (0.7–4.0)	0.19	1.6 (0.7–3.9)	0.256
	Both IRS 6–12	17	68.9 (52.2–85.7)		Reference		Reference	
Only grade 3 tumors		175						
Cytoplasmic	IRS 0	30	68.5 (51.5–85.4)		Reference		Reference	
	IRS 1–2	46	35.3 (21.1–49.4)	0.041	2.5 (1.1–5.5)	0.024	2.5 (1.1–5.7)	0.027
	IRS 3–4	51	61.9 (49.1–74.7)		1.2 (0.5–2.7)	0.693	1.1 (0.5–2.6)	0.782
	IRS 6–12	48	56.9 (45.3–68.5)		1.3 (0.6–2.7)	0.57	1.2 (0.5–2.9)	0.600
Nuclear	IRS 0	47	33.9 (24.8–43.1)	0.174	2.1 (1.0–4.2)	0.035	2.2 (1.1–4.6)	0.033
	IRS 1–2	46	60.5 (46.1–74.9)		1.4 (0.7–2.8)	0.389	1.6 (0.8–3.4)	0.225
	IRS 3–4	40	58.1 (43.5–72.6)		1.4 (0.6–2.8)	0.433	1.6 (0.7–3.6)	0.243
	IRS 6–12	42	63.6 (50.4–76.8)		Reference		Reference	
Combination cytoplasmic/nuclear	IRS 1–2/IRS 0	17	20.2 (12.6–27.8)	0.038	4.1 (1.3–12.7)	0.015	3.6 (1.1–12.0)	0.033
	Others	142	57.9 (49.5–66.3)		2.0 (0.8–5.1)	0.134	1.9 (0.7–5.0)	0.162
	Both IRS 6–12	16	73.0 (57.1–88.9)		Reference		Reference	

[a]Multivariate Cox regression analysis adjusted to tumor histology, tumor therapy, tumor grade, tumor stage, sex, and age; in grade 3 tumors adjusted to tumor histology, tumor therapy, tumor stage, sex, and age. The differences between univariate analyses are determined by censored cases before the events occurred in the groups (what is considered directly in the Kaplan-Meier analysis), and in the Kaplan-Meier analysis, the calculation covers all of the groups in one step, whereas in the univariate Cox regression analysis, it compares single groups with a reference group.
[b]Patients in Table 4 are the same as in Table 3 but one patient less where no PFS was recorded.

ing showed tumor progression on average after 20.2 months, whereas the group with a strong cytoplasmic and strong nuclear expression showed tumor progression on average after 73.0 months (57.1–88.9 months; P = 0.038; Figure 2B). In univariate and multivariate Cox regression hazard analyses, a 4.1-fold (P = 0.015) and a 3.6-fold (P = 0.033) increased risk of tumor progression for the weak cytoplasmic and negative nuclear staining group compared with the strong cytoplasmic/nuclear group was detected (Table 4).

DISCUSSION

The aim of this study was to investigate Piwil 2 protein expression in BCa and analyze its association with clinicopathological and survival data. We found no association of Piwil 2 protein expression with clinicopathological parameters. This finding, however, is still being discussed in the recent literature. In accordance with our results, Piwil 2 expression was not significantly different between the parameters of sex, age, histological grade and Dukes stage in colon cancer (19). Likewise, Piwil 2 has been shown to be expressed in various stages of breast cancers and cervical cancers without any differences between tumor stages (20,21). In contrast, Piwil 2 was demonstrated to be related to patient age, tumor size, histological subtype, tumor stage and lymph node metastasis

in breast cancer (23). In colon cancer, Piwil 2 expression has been reported to be correlated with tumor stage and pathological tumor staging (TNM) (29). Furthermore, a study of gastric cancer demonstrated that the expression of the Piwil 2 protein was positively correlated with T stage, lymph node metastasis and clinical TNM (30), and a study of colorectal cancer reported associations with tumor grade, depth of invasion and perineural invasion (31). Additionally, in a study focusing on the etiology of breast cancer, the expression patterns of Piwil 2 were observed in both the cytoplasm and the nucleus of invasive and metastatic breast cancers, while a nuclear expression pattern was less common in breast

precancers. Therefore, the authors suggested that Piwil 2 may be a novel biomarker for breast cancer (18).

We showed for the first time that a weak cytoplasmic and a negative nuclear expression for Piwil 2 were significantly associated with an increased risk of tumor-related death and an increased risk of tumor progression. The combination of both the cytoplasmic and nuclear staining patterns resulted in an additive effect and a further increased risk of tumor-related death and tumor progression for the BCa patients with weak cytoplasmic and negative nuclear staining for Piwil 2. This result points toward different biological cellular functions of Piwil 2 in the cytoplasm and in the nucleus. All of the BCa patients in this study were reclassified as having high-grade tumors according to World Health Organization (WHO) classification from 2004 (32) Additionally, the tumors were also classified according to the former WHO classification from 1997, distinguishing the high-risk patients in grade 2 and grade 3. The reported association between weak cytoplasmic and negative nuclear staining for Piwil 2 and a poor disease-specific and PFS was more pronounced in grade 3 BCa patients than in the entire cohort. This finding suggests that Piwil 2 staining patterns may have a stronger prognostic impact in grade 3 patients, although expression patterns were not significantly different between both tumor grade groups.

Our finding that weak cytoplasmic and negative nuclear Piwil 2 staining were associated with poor disease-specific and PFS was somewhat surprising because in gastric cancer and colon cancer, increased Piwil 2 protein expression was associated with a poor prognosis (29,30) and in papillary thyroid carcinoma and colon cancer the increased Piwil 2 protein expression was associated with metastasis (29,33). However, on the mRNA level *PIWIL2* appeared to be downregulated in poorly differentiated prostate cancers compared with normal glands of the peripheral zone (34). Consistent with our IHC data, Nikpour *et al.*

describe a very weak or even absent *PIWIL2* mRNA expression in BCa or BCa cell lines (25). It is important to note that there are six different protein isoforms (full-length Piwil 2 and five Piwil 2-like [PL2L] proteins: PL2L80, PL2L60, PL2L50, PL2L42 and PL2L40) (22). Somewhat comparably, in testicular germ cell tumors Gainetdinov *et al.* identified the two protein isoforms PL2L80A and PL2L60A in addition to Piwi-like 2 (18). The antibody used for this study (22) could detect Piwil 2, PL2L80 and PL2L60 proteins but not the other isoforms. Differences in the staining patterns in various tumor entities could possibly originate from different isoforms or different epitopes that are recognized by the different Piwil 2 antibodies. However, comparable to mRNA expression, Piwil 2 protein could also show tumor entity–specific protein expression.

Which distinct functions could Piwil 2 have in the nucleus and in the cytoplasm of tumor cells? Piwil 2 has been shown to be expressed in (germ) stem cells and to play a role in murine and human spermatogenesis, mostly by silencing transposable elements (reviewed in [9]). However, expression of Piwil 2 in normal tissues has rarely been detected, but re-expression in different tumor entities, such as breast cancer, cervical cancer, gastric cancer, colon/colorectal cancer, testicular germ cell tumors, ovarian teratoma/dysgerminoma, gastrointestinal stromal tumors, renal cell carcinoma and endometrial carcinomas, has been extensively described (16) (reviewed in [9,24,34]). Piwil 2 inhibits apoptosis and promotes proliferation through activation of the Stat3/Bcl-XL pathway (16). This finding could be further strengthened by showing that Piwil2/Stat3/c-Src forms a trimeric protein complex. Stat3 is phosphorylated by c-Src and translocates to the nucleus. There, Stat3 binds to the *P53* promoter and represses its transcription, resulting in an inhibition of p53-mediated apoptosis (35). Furthermore, Piwil 2 can play different roles in chromatin organization and regulation (reviewed in [9]). The functions of Piwil 2's

Drosophila ancestor, Piwi, have been excellently reviewed recently (15). In *Drosophila*, with only one Piwi gene, Piwi imprints repressive methylation marks on histone H3 lysine 9 (H3K9) in hetero- and euchromatin (together with heterochromatin protein 1A [HP1a] and histone methyltransferase Su(var)3-9), which inhibits RNA Pol II transcription and silences target genes (reviewed in [15]). In human cells, transfection of *PIWIL4* induced H3K9 methylation at the tumor suppressor locus p16^{Ink4a} (CDKN2A) (36). *PIWIL1* (*HIWI*) transfection in murine mesenchymal stem cells induced DNA methylation and also silencing of cyclin-dependent kinase inhibitors (CDKIs) (37). Furthermore, reduction of an importin-α family member (Kpn7) resulted in a reduction of Piwil 2 and downregulation of epigenetic modifications (including repressive marks on histone H3K27me3) in mice (38). However, it is not yet known if Piwil 2 also plays a role in histone or DNA methylation in humans. However, a role for Piwil 2 in chromatin regulation has been described. Piwil 2 mediates chromatin relaxation through regulating histone acetylation and in this way Piwil 2 is essential for DNA repair. Especially after cisplatin treatment, Piwil 2 may favor the removal of cisplatin-induced DNA intrastrand crosslinks, which is one mechanism for cisplatin resistance of tumor cells (14,33) However, chromatin relaxation may play different roles depending on the exact timing during tumor initiation and progression. High Piwil 2 expression may activate genes during initial tumorigenesis, which is in accordance with the expression of Piwil 2 in precancerous but not normal hematopoietic stem cells (39). However, a high expression of Piwil 2 during cisplatin treatment may allow greater accessibility for chemotherapeutic treatment and vice versa without expression in the nucleus, as detected in our study, and may prevent an effective cisplatin treatment. An elevated expression of Piwil 2 after cisplatin therapy could result in enhanced DNA damage repair and a diminished therapeutic re-

sponse (14). Recently, it was shown that Piwil 2 also plays a role in resistance to Fas-mediated apoptosis, and Piwil 2 can affect p53 phosphorylation in tumor cells, suggesting a novel mechanism of Piwil 2 in apoptosis and supporting the hypothesis that Piwil 2 plays an active role in tumorigenesis (40). Taken together, Piwil proteins are localized in the nucleus and act as epigenetic regulators and regulators of transposon activity. They are localized in the cytoplasm as regulators of RNA stability and translation (reviewed in [41]), but a clear distinction between the roles of Piwil 2 proteins in the different compartments still needs further investigation.

Recently, Piwil 1 expression has been shown for the first time in the mitochondria (and in the nucleus) of neuroblastoma cells (42). Interestingly, in the parasite *Leishmania*, the Piwi analogue localizes to its single mitochondria, suggesting a functional role in mitochondria for resistance to apoptosis (41).

In summary, we showed that the Piwil 2 protein staining patterns in the cytoplasm (weak) and in the nucleus (negative) of tumor cells are associated with poor disease-specific and with short PFS for cystectomy and chemotherapy-treated BCa patients. The combination of both staining patterns was associated with an additive effect on both outcomes that was even more pronounced in the subgroup of grade 3 BCa patients. Our results suggest that Piwil 2 plays a role in the biological tumor behavior of these BCa and that it has the potential to be used as prognostic marker for high-risk BCa patients.

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DISCLOSURE

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